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14. ABSTRACT
The purpose of this research is to investigate the role of a chromatin-modifying enzyme, called EZH2, in breast cancer epigenetics and to develop strategies to identify chemical inhibitors of this enzyme. EZH2 is a histone methyltransferase which modifies lysine-27 of histone H3, an epigenetic mark which is generally linked to gene silencing and is implicated in tumor suppressor silencing during breast cancer progression. Progress on this project includes: 1) Identification of target genes that are directly silenced by EZH2 in breast cancer cells and 2) Mapping of EZH2 binding sites within the chromatin of one such target gene. This mapping defines subregions of regulatory DNA likely to contain response elements that mediate EZH2 silencing. The delimitation and characterization of an EZH2 response element is required in the plan for engineering a breast cancer cell-based bioassay to screen for EZH2 inhibitors. These inhibitors provide important drug compounds to test as part of emerging epigenetic therapies to combat cancer.

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Introduction

This research project is to investigate a chromatin-modifying enzyme, called EZH2, which is implicated in epigenetic modifications that contribute to breast cancer progression (Bracken et al. 2003; Kleer et al. 2003; Raaphorst et al. 2003; Cha et al. 2005; Bachmann et al. 2006; Collett et al. 2006; Ding et al. 2006). EZH2 is a histone methyltransferase that modifies histone H3 on lysine-27 (Cao et al. 2002; Kuzmichev et al. 2002), which is a chromatin modification linked to gene silencing (Cao and Zhang 2004). The purposes of this research are to 1) reveal basic mechanisms and consequences of EZH2 function in breast cancer cells and 2) to develop a breast cancer cell line-based bioassay to screen for inhibitors of the EZH2 histone methyltransferase. This research entails identification and analysis of individual target genes that are silenced by EZH2 in breast cancer cells, the mapping and characterization of DNA elements within these genes that mediate EZH2 silencing, and the exploitation of these EZH2 response elements to engineer cell lines that enable identification of small molecule inhibitors of EZH2.

Body

The body of this Annual Progress Report is organized with respect to individual Tasks within the Statement of Work (SOW).

Task I-2A: To identify target genes that are silenced by EZH2 in breast cancer cell lines

A list of about a dozen candidate EZH2 target genes was compiled based upon expression microarray and chromatin IP-microarray (ChIP-Chip) data generated previously by others in studies using primarily non-mammary cell types (Kirmizis et al. 2004; Bracken et al. 2006; Squazzo et al. 2006). The main objective was to identify target genes that are directly silenced by EZH2 in the breast cancer cell lines SKBR3 and MCF7. To accomplish this, we first employed RNA interference (RNAi) to reduce EZH2 levels in the cells followed by RT-PCR to measure changes in target gene expression. Figure 1 shows that we can achieve 80-90% reduction of EZH2 protein levels by RNAi using a pool of double-stranded oligonucleotides targeted against EZH2. As expected, global levels of tri-methylated histone H3 on K27, which is deposited by EZH2, are also reduced when EZH2 is depleted (Fig. 1). Quantitative (real-

time) PCR analysis revealed that the candidate target genes CCND2 and MYT1 are de-repressed upon EZH2 knockdown (Fig. 2), suggesting that these target genes are silenced in breast cancer cells. CCND2 encodes the cell cycle regulatory protein cyclin D2 and MYT1 encodes a transcription factor. Similar RT-PCR results were obtained for these two target genes in SKBR3 and MCF7 cells.

We then performed chromatin IP studies to determine if EZH2 associates directly with the CCND2 and MYT1 genes. Figure 3 shows that EZH2 is detected in both the promoter and upstream regulatory regions of both CCND2 and MYT1, whereas EZH2 association is not seen with a negative control gene, GAPDH. Taken together with the RNAi results, these data indicate that CCND2 and MYT1 are directly silenced by EZH2 in breast cancer cell lines. We selected the CCND2 target gene for further analysis to identify and delimit DNA regulatory elements that mediate EZH2 silencing (Task II-1, below) and to assess the role of EZH2 in CpG DNA methylation of a target gene (Task I-2B, below).

Task I-2B: To determine if EZH2 and DNA methyltransferases collaborate in breast cancer cells

In addition to the intrinsic histone methyltransferase activity of EZH2 complexes (Cao et al. 2002; Kuzmichev et al. 2002), there is also evidence that EZH2 can partner with DNA methyltransferases to promote CpG DNA methylation (Vire et al. 2006). This is an important connection in cancer epigenetics since promoter hypermethylation on CpG is a common hallmark of cancer cells and is thought to play a key role in tumor suppressor loss (Jones and Baylin 2002). Having established CCND2 as a direct EZH2 target gene in breast cancer cells, we sought to determine if CpG methylation within the CCND2 upstream region depends upon EZH2. We performed methylation-specific PCR to test if methylation of CpG islands upstream of CCND2, previously shown to be methylated in breast cancer tissue (Evron et al. 2001), requires EZH2 function. Indeed, we found that EZH2 knockdown reduces genomic DNA methylation at a CpG island located 1.3 kb from the CCND2 start site in SKBR3 cells (Fig. 4, compare lanes 3 and 4 in MSP panel). This result suggests that EZH2 partnership with the DNA methylation machinery extends to CCND2 chromatin in breast cancer cells. Thus, this model target gene could be used to further investigate mechanisms that underlie linkage between histone H3-K27 methylation and CpG DNA methylation, which has been highlighted by several recent cancer epigenetics studies (Ohm et al. 2007; Schlesinger et al. 2007; Widschwendter et al. 2007).

Task II-1: To identify and test DNA fragments from EZH2 target genes that can mediate EZH2 silencing (EZH2 response elements)

To identify and delimit potential EZH2 response elements within the CCND2 regulatory region, we have first pursued high-resolution chromatin IP tests to determine where EZH2 physically associates with CCND2 chromatin in SKBR3 cells. Upon testing for EZH2 binding throughout the 4 kb upstream region of CCND2, we found that there is a major peak of EZH2 association from -1.6 to -3.2 kb and a second peak at the promoter/start site region (Fig. 5, top panel). Thus, we hypothesize that one or both of these regions may contain an EZH2 response element. To obtain further clues about potentially relevant DNA sites within these regions, we also mapped the distributions of the YY1 and OCT4 transcription factors in this same CCND2 region by chromatin IP. Although the general mechanisms that recruit EZH2 to chromatin sites in mammary cells are largely unknown, these two DNA-binding proteins have been implicated in EZH2 targeting in studies on embryonic stem cells and muscle cells (Caretti et al. 2004; Squazzo et al. 2006; Endoh et al. 2008). Therefore, they are good candidates to investigate in our system. Encouragingly, we find that both the YY1 and OCT4 distributions coincide with the EZH2 distribution in CCND2 chromatin (Fig. 5, second and third panels). We will next perform YY1 and OCT4 knockdown in these cells to determine if either factor is functionally required for EZH2 association. If we find that either YY1 or OCT4 is needed to target EZH2 to the CCND2 gene, then the distributions of their consensus DNA-binding sites can be used to help pinpoint locations of potential EZH2 response elements within CCND2 upstream DNA.

In parallel with the chromatin IP mapping, we have begun to develop a functional assay for EZH2 silencing in breast cancer cells. Our initial approach is to create luciferase reporter gene constructs for transient transfection assays. Figure 6 shows several reporter contructs that we have generated; each contains an intact CCND2 upstream region fused to luciferase or just the CCND2 promoter or upstream region in combination with the SV40 enhancer or promoter to drive expression. These constructs have been generated using the pGL3 series of luciferase reporter vectors (Promega). We will transfect these reporter constructs, plus a reference Renilla luciferase vector, either in presence or absence of a co-transfected EZH2 expression vector. EZH2 silencing in this assay will be assessed by the decline in luciferase levels upon EZH2 co-transfection. We will use MCF10A mammary cells, since endogenous EZH2 levels are lower here than in SKBR3 or MCF7 cells. If we achieve significant reporter

silencing in these transient assays, then we will test if this depends upon EZH2 histone methyltransferase activity using an enzyme-dead EZH2 catalytic site mutant (Croonquist and Van Ness 2005). If we are unable to achieve robust EZH2-mediated silencing in this type of assay, then we will pursue alternative assay systems that use chromosomally integrated rather than transiently introduced reporter constructs. Once a functional assay for EZH2 silencing in mammary cells is achieved, then we will use this assay to delimit and characterize EZH2 response elements. An assay system that recapitulates robust EZH2 silencing is needed to develop a breast cancer cell line-based bioassay for EZH2 inhibitors, as envisioned in Task II of our Research Plan.

Key Research Accomplishments

1) Identification of target genes that are directly silenced by EZH2 in breast cancer cells

2) Evidence that the linkage between EZH2 function and CpG DNA methylation extends to the CCND2 target gene in breast cancer cells

3) Mapping of EZH2, YY1, and OCT4 sites of association within the CCND2 target gene in breast cancer cells

Reportable Outcomes

1) Abstract: Era of Hope Meeting, June 2008, DOD Breast Cancer Research Program (attached as Appendix Item 1).

2) Review Article: Simon, J.A and Lange, C.A. (2008). Roles of the EZH2 histone methyltransferase in cancer epigenetics. ***Mutation Research***, in press, for Special Issue on "Epigenetics of development and human disease."
(attached as Appendix Item 2).

Conclusion

This research has identified individual EZH2 target genes in breast cancer cells, which can now be exploited in further studies to identify DNA elements responsible for gene silencing by EZH2. The identification and harnessing of these EZH2 response elements provide a key component of our planned strategy for isolating small molecule inhibitors of the EZH2 chromatin-modifying enzyme. Such inhibitors provide important lead compounds for the development and optimization of potential therapeutics that block EZH2 function. These inhibitors, and their derivatives, may find use in emerging strategies to combat cancer progression through drugs that alter epigenetic states of genomes in cancer cells (Egger et al. 2004; Lyko and Brown 2005; Yoo and Jones 2006).

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Appendices

Item 1) Abstract: Era of Hope Meeting, June 2008, DOD Breast Cancer Research Program

Item 2) Review Article: Simon, J.A and Lange, C.A. (2008). Roles of the EZH2 histone methyltransferase in cancer epigenetics. ***Mutation Research***, in press, for Special Issue on "Epigenetics of development and human disease."

Supporting Data

Figures 1-6, which accompany the Body of the Progress Report, are appended below.

Appendix 1

Abstract: Era of Hope Meeting, June 2008, DOD Breast Cancer Research Program

Title: Analysis of EZH2 function and target gene silencing in breast cancer cells

Authors: Liangjun Wang, Aswathy K. Nair, Carol A. Lange and Jeffrey A. Simon

EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which is a highly conserved chromatin-modifying enzyme complex. PRC2 methylates histone H3 on lysine 27, which is a repressive chromatin mark that contributes to gene silencing. Recently, EZH2 has been linked to aggressive forms of breast cancer as well as other cancers. Analyses of patient samples shows that abnormally elevated EZH2 levels correlate significantly with invasiveness and increased proliferation rates of breast carcinomas (Kleer et al. 2003; PNAS 100:11606). In addition, EZH2 over-expression in breast epithelial cells causes anchorage-independent growth and increased cell invasiveness in vitro and injection of EZH2 over-expressing cells into mammary tissue causes tumor production in mice (Kleer et al. 2003; PNAS 100:11606; Cha et al. 2005; Science 310:306). EZH2 has also been functionally linked to CpG DNA methylation (Vire et al. 2006; Nature 439:871). Tumor suppressor genes are frequently hypermethylated on CpG and silenced during breast cancer progression.

To address mechanisms by which EZH2 over-abundance could alter cellular phenotypes, it is important to identify and study regulatory regions of target genes that undergo EZH2-dependent chromatin changes in mammary cells. After screening EZH2 target genes identified in non-mammary cell types (Kirmizis et al. 2004; Genes Dev 18:1592; Squazzo et al. 2006; Genome Res 16:890), we found that the transcription factor MYT1 and cyclin D2 (CCND2) are direct EZH2 target genes in breast cancer cells. Depletion of EZH2 by RNAi in SKBR3 cells derepresses MYT1 by about 20-fold and CCND2 by about 5-fold. These EZH2 RNAi-treated cells also show decreased global levels of trimethylation on histone H3-K27. Chromatin immuneprecipitation (ChIP) assays show that EZH2 binds directly to the promoter and upstream regulatory regions of MYT1 and CCND2. We mapped EZH2 distribution in CCND2 upstream DNA to a region from -1.3 kb to -3.2 kb, which defines a putative EZH2 response element. In agreement with a link between EZH2 and DNA methylation, we found that EZH2 knockdown reduces levels of CpG methylation in this CCND2 region.

PRC2 often functions together with other Polycomb proteins in target gene silencing. In the *Drosophila* system, a DNA-binding protein called PHO helps recruit PRC2 to target genes, and histone methylation by PRC2 is thought to help recruit Polycomb repressive complex 1 (PRC1). We used ChIPs to address if the human homologs of these factors cooperate with PRC2 in SKBR3 cells. We find that YY1, the human homolog of PHO, and BMI-1, a subunit of human PRC1, are both recruited to the CCND2 upstream region where they tightly colocalize with EZH2. These results suggest that conserved Polycomb components cooperate in chromatin silencing at the CCND2 locus in breast cancer cells. In the short term, we plan to perform functional assays to delimit the EZH2 response element(s) in this region. In the long term, we wish to fuse EZH2 response elements to reporter genes to identify EZH2 chemical inhibitors using cell-based transcription readout screens. The identification of EZH2 inhibitors may provide new drug leads for development of anti-cancer therapeutics.

Appendix 2:

Mutation Research, in press

Roles of the EZH2 histone methyltransferase in cancer epigenetics

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Abstract

EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which is a highly conserved histone methyltransferase that targets lysine-27 of histone H3. This methylated H3-K27 chromatin mark is commonly associated with silencing of differentiation genes in organisms ranging from plants to flies to humans. Studies on human tumors show that EZH2 is frequently over-expressed in a wide variety of cancerous tissue types, including prostate and breast. Although the mechanistic contributions of EZH2 to cancer progression are not yet determined, functional links between EZH2-mediated histone methylation and DNA methylation suggest partnership with the gene silencing machinery implicated in tumor suppressor loss. Here we review the the basic molecular biology of EZH2 and the findings that implicate EZH2 in different cancers. We also discuss EZH2 connections to other silencing enzymes, such as DNA methyltransferases and histone deacetylases, and we consider progress on deciphering mechanistic consequences of EZH2 overabundance and its potential roles in tumorigenesis. Finally, we review recent findings that link EZH2 roles in stem cells and cancer, and we consider prospects for integrating EZH2 blockade into strategies for developing epigenetic therapies.

1. Introduction

Chromatin changes have long been associated with cancer (reviewed in [1-5]). The most well-characterized alteration is CpG DNA hypermethylation which often accumulates in promoter regions of tumor suppressor genes, thereby contributing to tumor suppressor loss through epigenetic silencing [1]. CpG hypermethylation at specific loci is so commonly observed in breast, prostate, and a wide variety of other cancers that this epigenetic alteration is considered a molecular marker of these diseases [6]. In addition to DNA methylation, epigenetic modification states of histones are also implicated in oncogenesis [2,7,8]. Particular global patterns of acetylation and methylation of histones H3 and H4 are associated with multiple cancer types and, in the case of prostate cancer, these modification patterns distinguish disease subtypes and can predict patient outcome [9,10]. These and other findings promote an emerging view that epigenetic changes in the cancer cell genome may contribute just as significantly to disease progression as do genetic alterations to DNA sequence [1,7,11]. However, there is a crucial difference between genetic and epigenetic alterations, which has important implications for development of cancer treatments. Once the DNA sequence is changed by mutation, it is difficult to restore the gene or counteract the altered gene product.

However, epigenetic changes can potentially be reversed with inhibitors that block the relevant chromatin-modifying enzymes. Thus, it is important to identify mechanisms of epigenetic enzymes in cancer cells with an eventual goal of developing strategies to impede their undesired activities.

2. Molecular biology of the EZH2 histone methyltransferase

Among the histone modifications associated with gene silencing and cancer, much has been learned recently about the enzymes responsible for methylation of histone lysine residues [2,8,12]. Here we focus on one of these histone methyltransferases, called Polycomb repressive complex 2 (PRC2), which is the major enzyme that methylates lysine-27 of histone H3 (H3-K27). When PRC2 methylates this residue, it can add up to three methyl groups to the ε-amino group of the lysine side chain. The tri-methylated form of H3-K27 is currently viewed as the predominant form that conveys biological function in vivo. Thus, referral here to methyl-H3-K27 indicates the tri-methylated form, unless stated otherwise.

PRC2 was initially purified and characterized from human cells and *Drosophila* embryos [13-16]. As depicted in Fig. 1A, human and fly PRC2 have very similar core subunit compositions. They each contain a conserved catalytic subunit, EZH2 in humans or E(Z) in flies, which contains the signature SET domain that provides the methyltransferase active site [17]. Structure determinations of other SET domains have revealed an unusual "thread-the-needle" structure, called a pseudoknot ([18,19] for reviews). The pseudoknot is formed by juxtaposition of two conserved peptide motifs within the SET domain, with one peptide inserted through the loop created by the other. These structures show that the substrate lysine and methyl donor cofactor bind opposite sides of the SET domain with their binding pockets connected by an interior channel that aligns the reactive groups for methyl transfer. However, the EZH2/E(Z) subunit lacks enzyme function on its own. Instead, EZH2/E(Z) must be complexed with at least two of its noncatalytic partners, EED/ESC and SUZ12, to attain robust histone methyltransferase activity [20-24].

Fig. 1B displays the domain organization of EZH2. Both the C-terminal SET domain and the adjacent cysteine-rich CXC domain are required for histone methyltransferase activity [13-15,20]. As also indicated in Fig. 1B, additional N-terminal domains provide binding sites for assembly with the required partner subunits. PRC2 enzyme function can also be influenced by another associated component, called PHF1 in human cells and PCL in flies. Although PHF1/PCL is not a core subunit of PRC2 (Fig. 1A), its association with the complex can

stimulate PRC2 enzyme activity and/or influence its recruitment to target genes in vivo [25-28]. The highly collaborative nature of the PRC2 enzyme complex, with multiple partners and inputs needed for function, is a key feature to consider in developing strategies for inhibition. That is, besides the catalytic site housed within the EZH2 SET domain, there are potentially many surfaces and binding pockets that could provide useful targets for binding inhibitory molecules.

A common biological function of PRC2 is transcriptional silencing of differentiation genes. Indeed, the role of PRC2 subunits in silencing *Drosophila* Hox transcription factors has long been recognized [29,30]. We now appreciate, through genome-wide studies (see below), that frequent PRC2 targets are transcription factors and signalling components with key roles in cell fate decisions in a wide variety of organisms. PRC2 and H3-K27 methylation are also implicated in mammalian X-chromosome inactivation [31] and the *C. elegans* version of PRC2 methylates H3-K27 and functions in germline silencing [32]. Plant versions of PRC2 deposit K27 methylation to regulate key events in seed and flower development [33]. This remarkable conservation of PRC2, including its catalytic EZH2 subunit, indicates an ancient strategy for chromatin silencing that deploys H3-K27 methylation as a repressive mark. Although the underlying mechanisms remain to be determined, there is ample evidence that disruption of this epigenetic silencing system contributes to oncogenesis.

3. EZH2 overabundance in cancer tissues

Since the basic discovery that EZH2 functions as a chromatin-modifying enzyme, many reports have appeared that link EZH2 to the altered properties of cancer cells. The common finding is that EZH2 levels are abnormally elevated in cancer tissues versus corresponding normal tissues, with the highest EZH2 levels correlating with advanced stages of disease and poor prognosis. In some cases, EZH2 overabundance is paralleled by amplification of the EZH2 gene. Table 1 provides a compilation of studies that report EZH2 overabundance in tissue samples from patients with different types of cancer. Among these, altered EZH2 levels have been most extensively documented in prostate and breast cancer. The functional consequences of EZH2 over-expression, which may include hypersilencing of genes that promote differentiation and restrain proliferation, are discussed in Sections 4-6 below.

3.1 EZH2 in prostate cancer

One of the earliest reports was a gene profiling study where EZH2 was scored as the most significant gene up-regulated in metastatic prostate cancer compared to clinically localized prostate cancer [34]. This study also showed that loss of EZH2 inhibits growth of prostate cancer cells. Similar requirements for EZH2 in proliferation of other cell types have been described [35,36]. Significantly, EZH2 over-expression in prostate cell lines led to silencing of a discrete set of >100 target genes, which was dependent upon an intact SET domain [34]. Thus, this work suggested that EZH2 overabundance alters the genomic expression program through chromatin hypersilencing. Statistical analysis also revealed that EZH2 levels could provide a valuable prognostic indicator of patient outcome [34] and subsequent studies have described the prognostic value of combined sets of prostate markers that include EZH2 overabundance [37,38]. More recently, a Polycomb repression "signature", consisting of a cohort of 14 repressed EZH2 target genes, has been described as a tool for predicting prostate and breast cancer patient outcomes [39].

3.2 EZH2 in breast cancer

Analyses of patient samples significantly correlates abnormally elevated EZH2 levels with invasiveness and increased proliferation rates of breast carcinomas [35,40-42]. These studies also emphasize EZH2 as a prognostic indicator of outcome in breast cancer patients [40,42], reflecting the significant association of high EZH2 levels with aggressive forms of the disease. EZH2 accumulation may even provide an early molecular marker to detect precancerous changes in histologically normal mammary tissue [43]. To address cause-and-effect relationships between EZH2 function and oncogenesis, the consequences of engineered EZH2 over-expression in mammary cells have been examined. These studies have shown that EZH2 over-abundance in breast epithelial cells causes anchorage-independent growth and increased cell invasiveness in vitro [42] and EZH2 over-expressing cells are tumorigenic when injected into the mammary fat pads of nude mice [44]. Another study, using myeloma cells, showed that the oncogenic properties of EZH2 in mice correlate with its histone methyltransferase activity [36].

3.3 Cancer links to other PRC2 subunits

Less is known about cancer-associated alterations in PRC2 subunits besides EZH2. EED over-expression in human cancers has not been widely reported and the studies that first documented EZH2 over-expression in prostate and breast cancer revealed unchanged EED

levels in the same patient samples [34,42]. However, elevated abundance of a particular EED isoform, EED2, has been described for breast and colon tumors ([45]; see below). There are also examples of SUZ12 alterations in cancer tissues (Table 1) including over-expression in colon, breast and liver tumors [45-47]. In addition, SUZ12 (also called JJAZ1) is implicated in endometrial cancer since a chromosome rearrangement creating a SUZ12 fusion protein is frequently associated with endometrial stromal tumors [48]. Finally, over-expression of PCL3, which is a homolog of the PRC2-associated protein, PHF1 (Fig. 1A), is also associated with many cancers including colon, skin, lung and liver [49]. Since consequences of EZH2 overabundance in cancer cells are still emerging, it is an open question if excessive levels of these noncatalytic partners work through similar mechanisms.

4. Collaboration of epigenetic silencing enzymes: Functional links between EZH2 histone methyltransferase, DNA methyltransferases, and histone deacetylases

4.1 EZH2 links to DNA methylation

Polycomb silencing and DNA methylation have often been considered biochemically independent gene silencing systems. In agreement with this, *Drosophila* and *C. elegans* deploy PRC2 and H3-K27 methylation in silencing yet little or no DNA methylation is detected in their chromatin. However, recent studies in human cells, showing that EZH2 and DNA methyltransferases (DNMTs) are physically and functionally linked, have fundamentally altered this outlook and prompted important new models in cancer epigenetics. The key initial study [50] showed that PRC2 subunits (EZH2 and EED) co-immuneprecipitate with all three human DNMTs and that silencing of certain target genes requires both EZH2 and DNMTs. Significantly, RNAi knockdown in osteosarcoma cells showed that EZH2 is needed for DNMT binding and CpG methylation of target genes but, conversely, DNMTs are not needed for EZH2 chromatin association [50]. These data suggest a pathway where EZH2 acts upstream of DNMTs to methylate and silence target chromatin (Fig. 2). It is not yet clear if DNMTs are recruited primarily by direct EZH2 contact, by the methyl-H3-K27 chromatin mark, and/or by other intermediary factors. The observation that wild-type EZH2, but not a mutant lacking the SET domain, could recruit DNMTs [50] implies that PRC2 catalytic function is involved. However, once a target gene becomes densely CpG hypermethylated, its maintained DNA methylation and silencing may no longer require EZH2 [51].

Subsequent studies expanded the EZH2-DNA methylation link by comparing chromatin states in cancer cells versus normal cells and by investigating many more target genes [52-54]. These gene profile comparisons reveal that EZH2 target genes, which display Me-H3-K27 in normal cells, are highly correlated with genes that become abnormally hypermethylated in cancer cells. Collectively, these studies suggest that EZH2 pre-marks certain genes to later become CpG hypermethylated during cellular transformation. Thus, genes that acquire Me-H3-K27 during normal development are somehow predisposed for DNA hypermethylation and conversion to "deep" silencing [7,52] in the presence of oncogenic cues. These cues presumably include abnormally high EZH2 levels but additional factors are likely involved. One group suggests that chromatin modifications on H3-K9 may also contribute during this transition [52]. Although much of this data relies on genes hypermethylated in colon cancer, similar findings were reported for other tumor types including prostate, liver, lung, ovarian and breast [53,54]. A recent study has also described functional connections between EZH2 and DNA methylation in acute promyelocytic leukemia [55].

4.2 EZH2 links to histone deacetylation

Physical and functional links between EZH2 and histone deacetylases (HDACs; [56,57]) predate the basic discovery that PRC2 has histone methyltransferase activity. In human cells, PRC2 can physically associate with HDACs 1 and 2 [14,57] and PRC2-mediated transcriptional silencing is impeded by the HDAC inhibitor TSA [34,57]. The sum of the biochemical data suggests that HDACs are not core subunits of PRC2 [12-16] but transient interactions likely still provide functional synergy between these silencing enzymes in vivo. The precise mechanisms of this synergy at target gene chromatin are not yet clear. As illustrated in Fig. 2, HDACs could deacetylate H3-K27 to make the ε-amino group available for methylation by PRC2. Alternatively, HDACs could deacetylate other histone lysines, such as H3-K9, H3-K14 or H4-K8, to adjust the local histone code for silencing. Taken together, functional links between EZH2, HDACs, and DNMTs contribute to an emerging view that all three types of epigenetic silencing machinery contribute to abnormal control of gene expression in cancer cells.

5. Towards deciphering EZH2 mechanisms in cancer cells

Although there is a large body of data implicating EZH2 in cancers of many types (Table 1), relatively little is known about molecular mechanisms of altered EZH2 function in cancer cells.

It is important to fully understand: 1) biochemical changes that affect PRC2 composition and/or activity in cancer cells, 2) how chromatin states and expression of EZH2 target genes are altered in cancer cells, and eventually 3) how this altered expression profile contributes to oncogenesis. Recent progress in these areas, as well as possible cytoplasmic EZH2 function, are discussed below. Since the main mechanistic question concerns the consequences of EZH2 over-expression, it is worth emphasizing that there is normally little EZH2 in adult differentiated tissues [34,35,42]. In contrast to widespread EZH2 roles in early mouse development [58,59], post-embryonic EZH2 expression is limited [60,61]. Even when detected in adult tissues, EZH2 is concentrated in undifferentiated progenitor cell populations, such as hematopoietic cells of the pro-B lymphocyte lineage [62]. Thus, EZH2 "over-expression" in cancer tissues may reflect inappropriate EZH2 accumulation in cell types that normally lack it as opposed to merely adjusting EZH2 levels upwards in cells where it normally functions.

5.1 Altered forms of PRC2 in cancer cells

Different forms of the EZH2 complex occur in human cells, which are distinguished by the particular EED subunit included [63]. Specifically, there are four EED isoforms, distinguished by N-terminal extensions of differing lengths, that are produced from alternative translation start sites [63]. Canonical PRC2 complexes contain the longest EED isoform, called EED1, whereas PRC3 and PRC4 contain the shorter EED isoforms. Significantly, the PRC4 variant of the histone methyltransferase complex is selectively enriched in cancer cells versus normal cells and PRC4 assembly is favored when EZH2 is over-expressed in a cell line [45]. PRC4 differs biochemically from the other EZH2 complexes in several ways: 1) it contains the second largest EED isoform, EED2, 2) it also contains the NAD-dependent histone deacetylase, SirT1, and 3) it prefers to methylate histone H1-K26 rather than H3-K27. Since SirT1 can deacetylate H1-K26, its role in PRC4 may be to prepare this H1 residue for methylation [45]. This study raises the key prospect that PRC2 is compositionally and functionally reconfigured in a cancer-specific context. Furthermore, the results suggest that H1 methylation, rather than H3, might be a critical EZH2-sponsored modification in cancer cells. However, little is yet known about in vivo functions of H1-K26 methylation and an independent study using recombinant EZH2 complexes failed to detect H1 methylation when EED isoforms were varied [64]. Additional studies on a wide array of tumor samples will be needed to assess if H1-K26 methylation and/or EED2 over-abundance are commonly observed during cancer progression. Intriguingly, a recent study finds that trimethyl-H3-K27 levels are *decreased* in breast, ovarian and

pancreatic cancer samples [65], which could reflect a shift in lysine substrate preference. The idea that cancer cells might preferentially deploy an altered form of PRC2 is clinically important because cancer-specific subunits or interactions could provide targets for specifically inhibiting aberrant PRC2 functions without adversely affecting normal roles.

5.2 Altered expression of EZH2 target genes: obligate silencing?

The predominant current view is that EZH2 functions in cancer as a dedicated transcriptional repressor that hypersilences an array of target genes, including tumor suppressor genes. This view is supported by the cohort of PRC2-repressed genes linked to poor outcome in prostate cancer patients [39] and the coincidence of EZH2 target genes and genes hypersilenced by DNA methylation in cancer, as discussed above [52-54]. Moreover, genome-wide studies revealed that only a very small percentage (<1% in F9 teratocarcinoma cells) of genes are simultaneously bound by PRC2 and RNA polymerase II [66,67], which also implies nearly universal PRC2 silencing. However, studies of EZH2 in other contexts have yielded occasional evidence for roles in target gene activation. Although the majority of responding genes were up-regulated after PRC2 knockdown in colon cancer cells, a substantial minority were down-regulated, including some direct PRC2 targets [46]. In human fibroblasts, PRC2 subunits were required for expression rather than silencing of proliferation genes [35] and a role for PRC2 in activating certain target genes in mouse ES cells has been considered [68]. Moreover, a recent report describes EZH2 as an activator of cell cycle control genes in breast cancer cells [69] and this activator function appears independent of the SET domain.

These varying results emphasize that basic transcriptional mechanisms of EZH2 in cancer remain an open question. Although most evidence favors a predominant EZH2 role in silencing, further work is needed to address alternatives. Indeed, a potential dual role for the *Drosophila* version of EZH2 in silencing and activation has long been considered [70,71].

A related issue concerns just what happens when excess EZH2 accumulates in a cancer cell. The simplest view might be that it assembles to create abnormally high levels of PRC2, which then hypermethylates H3-K27 and hypersilences target genes. This outcome assumes that SUZ12 and EED are present in sufficient quantities to partner with the extra EZH2. Alternatively, an imbalance of PRC2 subunits could lead to accumulation of unassembled EZH2 or trigger production of aberrant PRC2 subcomplexes. Since EZH2 enzyme function requires assembly with its partners, free EZH2 or catalytically inactive subcomplexes could act

as dominant-negatives that desilence rather than hypersilence target genes. Thus, like the PRC2 isoforms described above [45], it is important to address if and how shifts in PRC2 subunit stoichiometries might impact target gene responses in cancer cells.

5.3 EZH2 recruitment to target genes

Since none of the PRC2 subunits are sequence-specific DNA-binding proteins, it is not known how EZH2 histone methyltransferase is recruited to target genes. In *Drosophila*, Polycomb response elements (PREs) have been delimited using reporter assays and several DNA-binding proteins are implicated in recruiting PRC2 (reviewed in [72]). However, mammalian PREs have yet to be precisely defined. A good candidate for a mammalian PRC2-targeting factor is YY1, whose *Drosophila* homolog, pleiohomeotic (PHO), is the best-characterized recruiter of PRC2 in flies [73]. Indeed, YY1 is needed for H3-K27 methylation of target genes in muscle cells [74]. However, this role may be cell-type specific as there is little overlap between YY1 targets and PRC2 targets in mouse ES cells [66]. Instead, Oct4 has been implicated in PRC2 targeting in ES cells ([66,75]; see below). Another study, using promyelocytic leukemia cells, shows that PRC2 can be recruited to target loci through interaction with the PML-RARα fusion protein but not with wild-type RARα [55]. Finally, a long non-coding RNA has been implicated in targeting PRC2 to the human *HoxD* cluster [76]. More work is needed to define sequence elements and mechanisms that recruit PRC2 to target loci in mammals. Taken together, the current data suggest that recruitment factors are likely to vary in different cell types and contexts.

5.4 EZH2 silencing and partnership with PRC1

Numerous studies have established that PRC2 histone methyltransferase frequently partners with another Polycomb complex, called PRC1, to achieve silencing in many systems ([8,77] for reviews). This partnership in human cells is supported by genome-wide mapping, which reveals frequent co-occupancy of target genes by both PRC2 and PRC1 [78,79]. The core subunits of human PRC1 are Polycomb (PC), polyhomeotic (PH), the oncoprotein BMI-1, and RING1, with the precise composition varying due to alternative subunit family members [80]. The chromodomain of the PC subunit can bind to tri-methyl-H3-K27 [81], which has inspired models wherein PRC1 is recruited to target chromatin by affinity for the methyl mark deposited by PRC2 [12]. In support of this idea, studies in both human cells and *Drosophila* have found that PRC2 function is needed for PRC1 recruitment to target genes [15,78]. Other

models suggest that PRC1 interaction with K27-methylated nucleosomes is primarily to form intralocus chromatin loops that contribute to further histone modifications and silencing [72,77]. Despite these variations, it is worth emphasizing that PRC1 is commonly viewed as the direct executor of Polycomb silencing at many target genes. The actual Polycomb silencing mechanism(s), which may include blocks to nucleosome remodelling [80], chromatin compaction [82], histone H2A ubiquitylation [83] and/or blocks to transcription elongation [84-86] remain to be fully elucidated. More work is also needed to determine if the output of abnormal EZH2 function in cancer cells depends upon or is independent of PRC1 partnership. As illustrated in Fig. 2, (see also [7]), a PRC1-independent mechanism could feature PRC2-mediated histone methylation leading to permanent silencing by CpG DNA methylation.

5.5 EZH2 function at the actin cytoskeleton

Although the vast majority of work on EZH2 is focused on chromatin regulation, EZH2, SU(Z)12 and EED are also detected in the cytoplasm of mouse and human cells and the methyltransferase is implicated in controlling actin polymerization in response to cell signalling [87]. Correspondingly, EZH2 overabundance could affect cytoskeletal-based behaviors such as migration and invasion of cancer cells. Indeed, recent reports indicate that EZH2 over-accumulation in prostate cancer cell nuclei is paralleled by cytoplasmic overabundance and knockdown suggests that EZH2 influences invasiveness and F-actin polymerization in these cells [88,89]. Thus, nuclear and cytoplasmic functions could both contribute to EZH2-mediated alterations in cancer cells.

6. EZH2 functions in stem cell biology: connections to cancer

6.1 Differentiation gene silencing in stem cell maintenance

Genome-wide searches for PRC2 target genes have been performed by chromatin immuneprecipitation of EZH2 or SUZ12 coupled to genomic microarray hybridizations (ChIP-on-Chip). The first of these ChIP-on-Chip studies identified PRC2 target genes in colon cancer cells [46] and subsequent genome-wide searches have been conducted in human embryo fibroblasts [79], breast cancer cells [66], and both mouse and human embryonic stem cells [66,67,78]. A fundamental finding from these studies is that PRC2 target genes are highly enriched for transcription factors and signalling components that control cell differentiation. This preferential PRC2 role in developmental networks is also evident from genome-wide

searches in *Drosophila* [90-92]. Thus, the originally defined function of fly Polycomb proteins as silencers of Hox differentiation factors [93] has been expanded to include dozens of other differentiation factor targets such as members of the Gata, Sox, Fox, Pou and Pax transcription factor families and components in Wnt, TGF-ß, Notch, FGF and retinoic acid signalling [67,78,79]. Although these myriad factors function in many different tissues including neuronal, bone, muscle, blood and skin, a common role is in converting stem cell-like progenitors into more differentiated cell types within these and other lineages.

The analysis of PRC2 distribution and function in embryonic stem (ES) cells is particularly striking and informative [67,78]. Three transcription factors, Oct4, Sox2 and Nanog, play critical roles in programming ES cell gene expression to maintain pluripotency [94]. In general, these factors promote expression of proliferation genes and they silence differentiation genes. The very high correspondence between silenced genes bound by these three factors with those that also bind PRC2 [67,78] suggests that PRC2 is a key corepressor in ES cells. Indeed, loss of PRC2 derepresses ES cell differentiation genes [67,78] and knockdown of Oct4 disrupts PRC2 association with target genes [66,75]. Thus, PRC2 is implicated in ES cell self-renewal as an inhibitor of the differentiation program. This role could explain why ES cell lines cannot be derived from null EZH2 mouse embryos [59]. A similar PRC2 role is envisioned in progenitor cell types such as multipotential neuronal or hematopoietic cells; although the target genes vary in different lineages, the common function would be stem cell maintenance via PRC2 silencing of differentiation genes. Recent studies show that differentiation gene silencing in mouse ES cells also requires the PRC1 subunit, RING1 [75,95], which suggests that stem cell maintenance requires PRC2/PRC1 collaboration as seen in other examples of PcG silencing [8,77].

Intriguingly, the PRC2 reaction product, methyl-H3-K27, is part of a specialized chromatin state, termed a "bivalent domain," that marks the silenced ES cell differentiation genes [96]. This state features nucleosomes methylated on histone H3-K4 encompassed by larger expanses of chromatin bearing methylated H3-K27. Their simultaneous accumulation is unusual since methylated H3-K4 generally promotes activation whereas methylated H3-K27 leads to silencing. In this case, methyl-H3-K27 appears to "win out" since target genes in this state remain off. These bivalent domains could provide a sensitized state whereby crucial differentiation genes are kept silent but nevertheless poised for rapid activation in response to differentiation stimuli. For example, erasure of H3-K27 methylation by lysine-specific demethylases [97,98] could resolve bivalent domains to trigger gene activation.

6.2 EZH2, stem cell properties, and cancer

The many parallels between stem cells and tumor cells, including high proliferation rates and differentiation capacity, have prompted hypotheses that undifferentiated or dedifferentiated precursor cells may play key roles in oncogenesis. Growing evidence in favor of this "stem cell origin of cancer" hypothesis has been extensively reviewed [99-102]. In this context, the role of EZH2 in promoting self-renewal and impeding differentiation of ES cells suggests potentially similar roles during cancer progression. In molecular terms, tumor suppressor genes may resemble stem cell differentiation genes by featuring flexible chromatin states that are initially "transcription-ready" [52]. During oncogenesis, this plastic state could progress to permanent silencing, for example by further acquisition of DNA methylation [52-54]. The trigger for this proposed chromatin transition is not known but presumbly involves EZH2 over-expression. Since there is normally little EZH2 in differentiated adult tissues, EZH2 overabundance could shift expression profiles to promote a return to or reinforcement of a stem-cell like state. Remarkably, a direct link between poorly differentiated human tumors and the ES cell state is provided by a shared gene expression signature defined in part by PRC2 target genes and Oct4/Sox2/Nanog target genes [103]. Clearly, the pace of discovery at the intersection between stem cell biology, chromatin, and cancer epigenetics is accelerating. These rapidly expanding topics are nicely integrated in these recent review articles [7,11].

7. Towards epigenetic therapy including EZH2 blockade

The epigenetic silencing of tumor suppressor genes in cancer has inspired potential therapeutic strategies that use inhibitors of epigenetic enzymes ([4,104,105] for reviews). A goal of epigenetic therapy is to achieve pharmacological reactivation of abnormally silenced genes in cancer patients, which could arrest or even reverse processes contributing to tumorigenesis. There are many inhibitors available that target either DNMTs or HDACs and clinical trials are underway to assess these [104-108]. Since epigenetic enzymes often synergize in vivo, as discussed above, there is also great interest in testing combined inhibitor treatments that target more than one epigenetic enzyme. In cell and animal models, simultaneous disruption of DNMTs and HDACs has produced encouraging results on gene reactivation (reviewed in [4]). One of the principles to emerge is that DNA methylation appears to dominate silencing, such that sequential treatment with DNMT inhibitor followed by HDAC inhibitor is preferred for optimal gene reactivation [109,110]. Some early clinical trials are

beginning to test efficacies of combined DNMT/HDAC inhibitors in leukemia patients [107], with at least one study reporting reversal of DNA methylation and hematological improvement [111].

Similar to DNMTs and HDACs, EZH2 histone methyltransferase has emerged as a key target in potential epigenetic strategies. However, specific inhibitors of EZH2 histone methyltransferase have not yet been described. Although small molecule inhibitors of other histone methyltransferases are emerging [112], the most encouraging inhibitory agent of PRC2 reported so far is deazaneplanocin A (DZNep), which works through an indirect mechanism [113]. DZNep is an S-adenosylhomocysteine (Ado-Hcy) hydrolase inhibitor; it causes Ado-Hcy levels to rise, which blocks S-adenosylmethinone-dependent methyltransferases through by-product inhibition. Importantly, DZNep can deplete PRC2 subunits in breast cancer cell lines and reactivate PRC2-silenced genes [113]. However, since this type of inhibitor may affect many processes that require methyl transfer, there are concerns about its specificity as a potential therapeutic. Alternative strategies for designing specific PRC2 inhibitors would include targeting the EZH2 active site and/or surfaces for key subunit interactions. A recent report provides structural data on the interface that mediates EZH2-EED interaction in PRC2 [114]. A high-resolution structure for the EZH2 SET domain, which houses the methyltransferase active site, would profoundly influence design of small molecule inhibitors specific for PRC2. The availability of these histone methyltransferase inhibitors should expand the repertoire of new possibilities in combined epigenetic therapy.

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Figure Legends

Figure 1. Composition of PRC2 and domain organization of EZH2. A) The four core subunits of human PRC2 are EZH2, EED, SUZ12 and RbAp48 [14,15] and the corresponding homologous subunits in fly PRC2 are E(Z), ESC, SU(Z)12 and NURF55 [13,16]. EZH2/E(Z) is the catalytic subunit that contains a SET domain. PHF1/PCL is another Polycomb protein that

can associate with PRC2 to influence its activity and/or targeting [25-28]. B) Five functional domains in EZH2/E(Z) are depicted, with % identities between the human and fly versions indicated. The SET domain houses the histone methyltransferase active site and the CXC domain also contributes to activity. Robust methyltransferase requires EZH2 assembly with both EED/ESC and SUZ12, and domains required for binding these noncatalytic subunits are indicated [20-22,114].

Figure 2. Model for collaboration of epigenetic silencing enzymes. Target genes are initially silenced through histone H3-K27 methylation by PRC2. If K27 is pre-acetylated, then methylation of this residue may first require deacetylation by a histone deacetylase (HDAC), which are known to interact with PRC2 [14,57]. PRC2 may also recruit DNA methyltransferases (DNMTs) [50] which methylate CpG DNA of target genes, leading to a more permanently or deeply silenced chromatin state [52-54]. These chromatin modifications encompass many nucleosomes and CpG elements per target gene; for simplicity, only a single nucleosome and CpG element in an upstream regulatory region are shown. "Ac" denotes acetylation and "Me" denotes methylation.

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Table 1. Human cancers associated with over-expression of PRC2 subunits.

PRC2 subunit	Type of cancer	References
EZH2	Prostate	[34,37,38,41,115,116]
	Breast	[35,40-43,117]
	Lymphoma	[118-120]
	Myeloma	[36]
	Bladder	[121-123]
	Colon	[124]
	Skin	[41,125]
	Liver	[126]
	Endometrial	[41]
	Lung	[127]
	Gastric	[128]
SUZ12	Colon	[45-47]
	Breast	[45,47]
	Liver	[47]

Figure 1

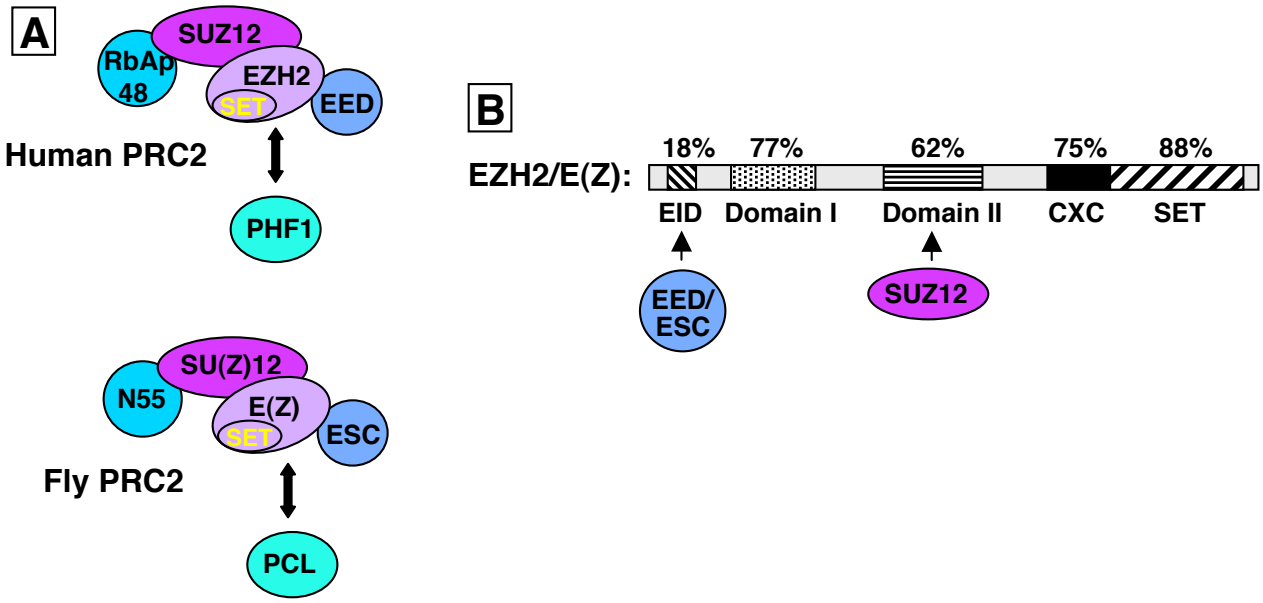
A
SUZ12
RbAp 48
EZH2
SET
EED
Human PRC2
PHF1
SU(Z)12
N55
E(Z)
SET
ESC
Fly PRC2
PCL
B
EZH2/E(Z):
18%
77%
62%
75%
88%
EID
Domain I
Domain II
CXC
SET
EED/ ESC
SUZ12

Figure 2

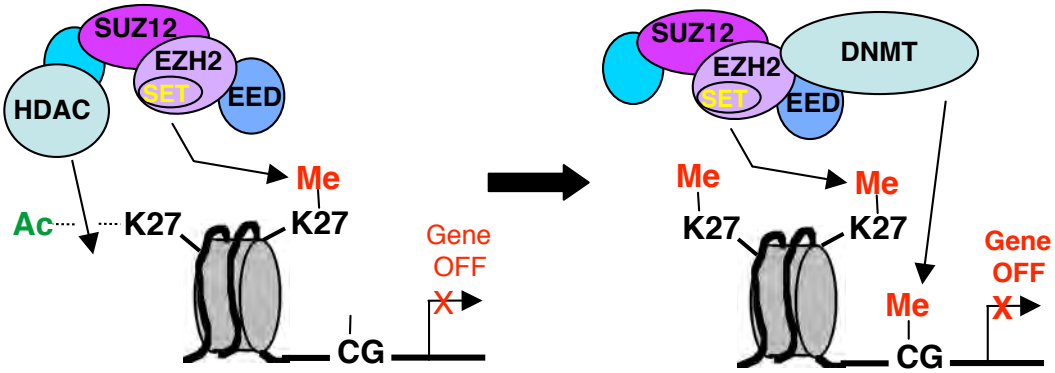
SUZ12
EZH2
SET
EED
HDAC
Me
Ac
K27
K27
CG
Gene
OFF
SUZ12
EZH2
SET
EED
DNMT
Me
Me
K27
K27
Me
CG
Gene
OFF

Figure 1

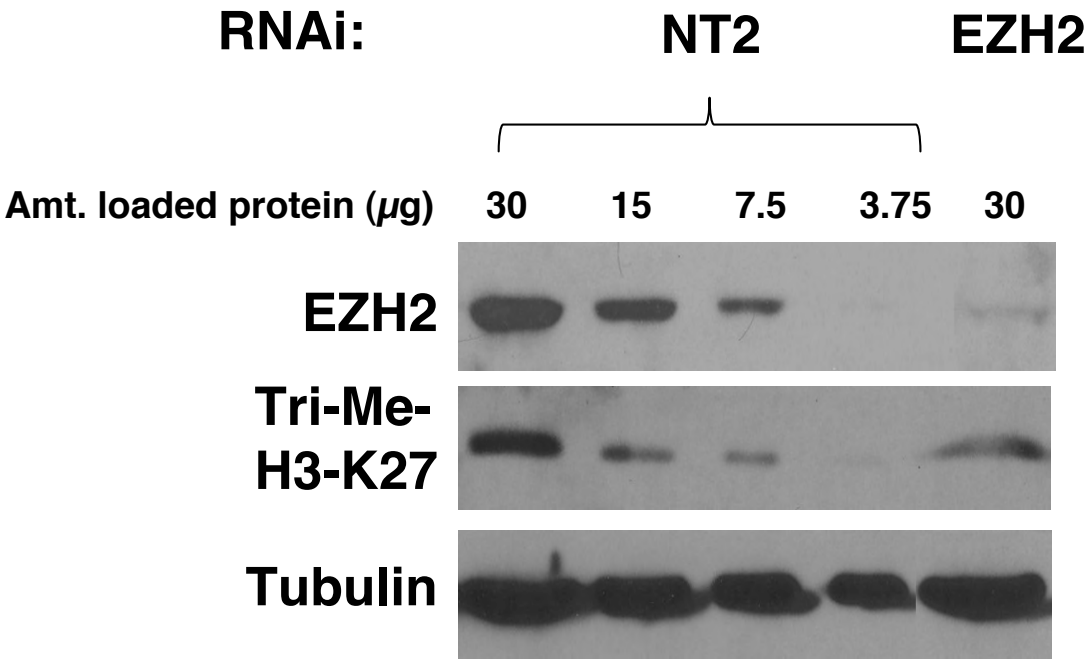

Figure 1. EZH2 knockdown in breast cancer cells by RNA interference. Panels show Western blots to detect EZH2, histone H3 trimethylated on K27, or tubulin (as a loading control) in protein extracts from SKBR3 cells. Samples were prepared from cells treated with a pool of double-stranded oligonucleotides to target depletion of EZH2 or with a non-target (NT2) pool of oligonucleotides to serve as negative control. Amounts of total protein loaded per lane are indicated. The EZH2 RNAi treatment reduces levels of EZH2 protein and trimethylated H3-K27 compared to nontargeted control.

Figure 2

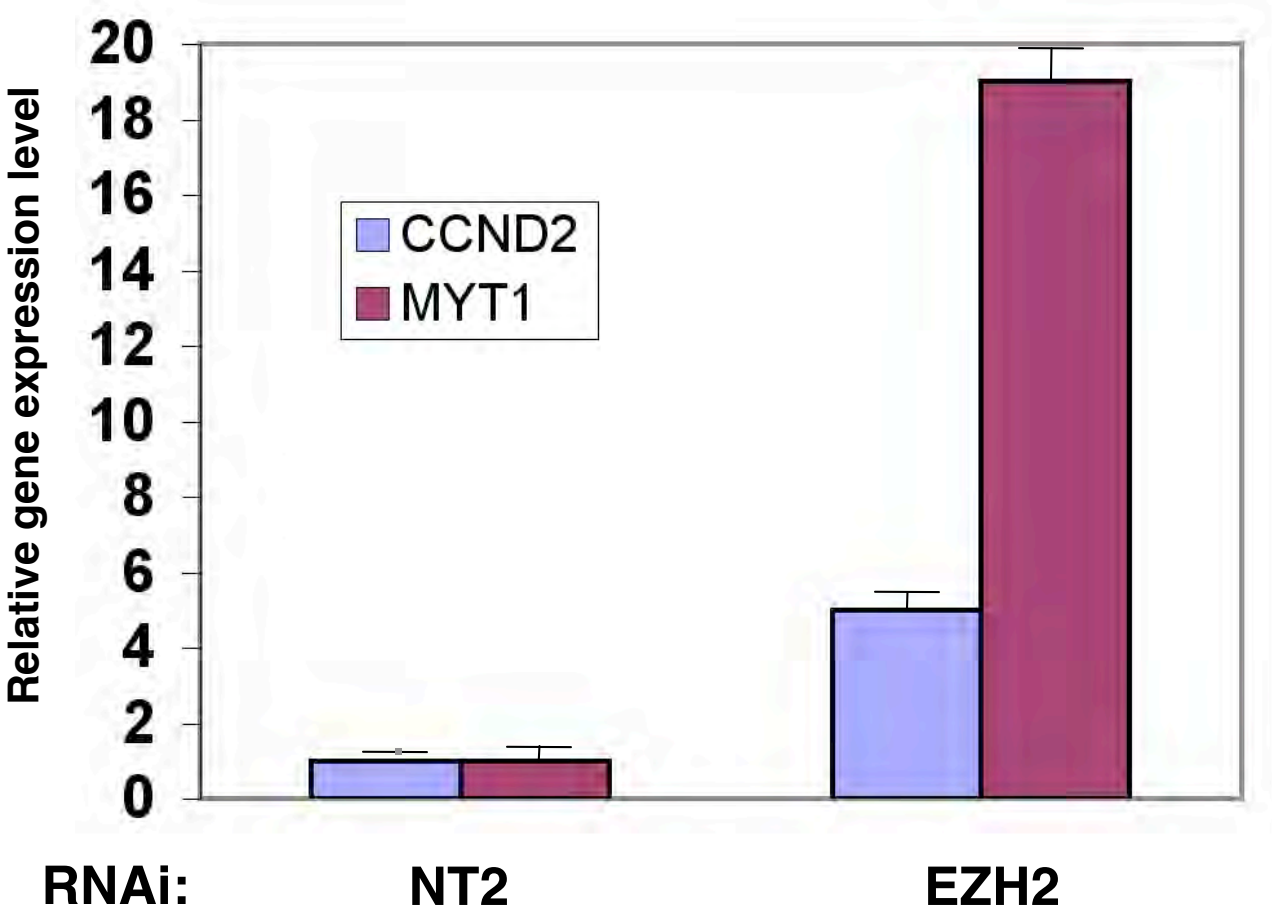

Figure 2. EZH2 knockdown derepresses the CCND2 and MYT1 target genes in breast cancer cells. Bar graphs depict relative abundance of CCND2 and MYT1 mRNAs as determined by quantitative (real-time) RT-PCR. RNA samples were prepared from SKBR3 cells treated with a pool of double-stranded oligonucleotides to deplete EZH2 or with a non-target (NT2) pool of oligonucleotides as negative control. Error bars represent standard deviations from triplicate samples.

Figure 3

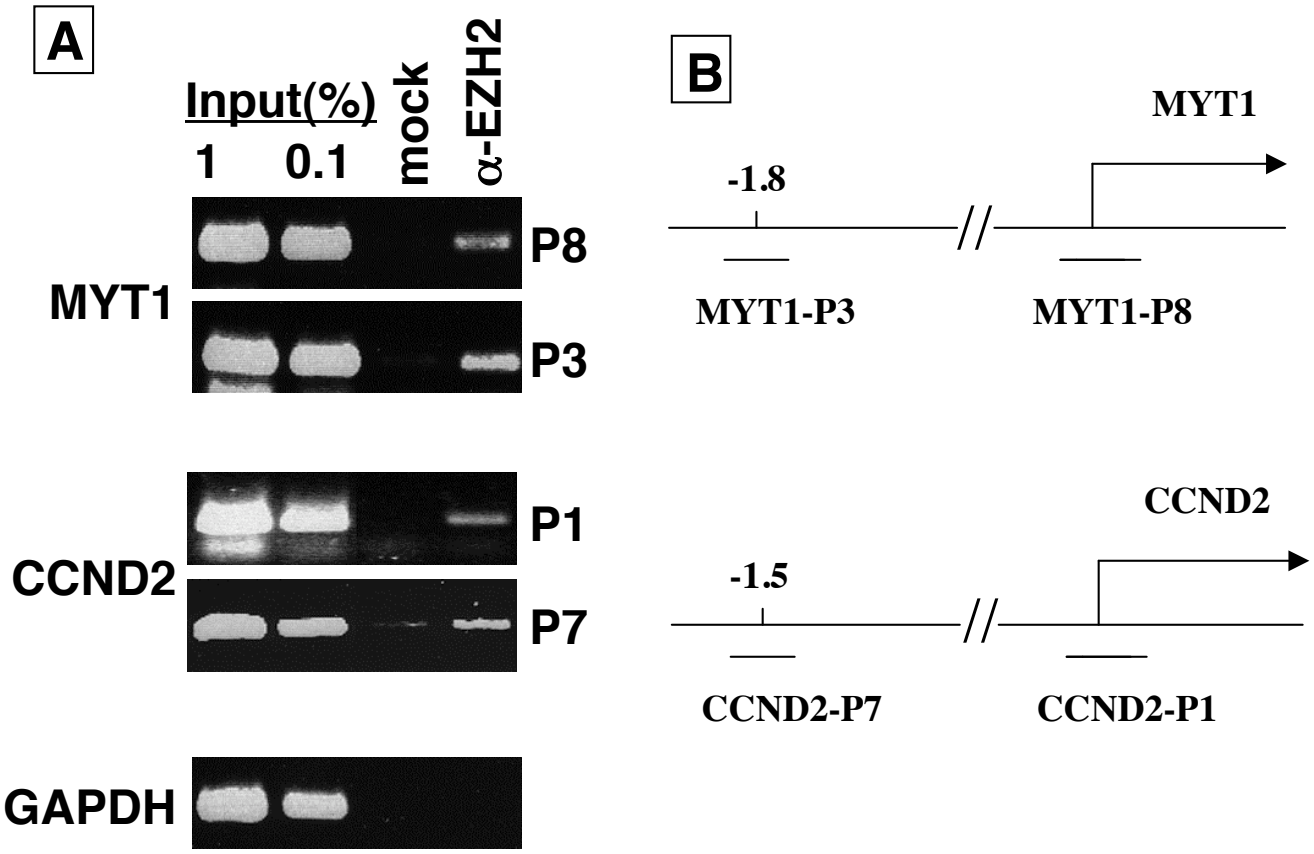

Figure 3. Chromatin immuneprecipitation detects direct EZH2 association with the MYT1 and CCND2 target genes in breast cancer cells. A) Chromatin IPs to detect EZH2 binding to MYT1 and CCND2 fragments from their respective promoter and upstream regions (shown in B) in SKBR3 cells. "Mock" indicates negative control immunoprecipitation lacking antibody and "input" samples contain PCR products amplified from indicated dilutions of genomic DNA. EZH2 associates with promoter and upstream regions of MYT1 and CCND2 but not with a negative control gene (GAPDH). In (B), upstream regions tested are located 1.5 to 1.8 kb from the transcription start sites, which are denoted by arrows.

Figure 4

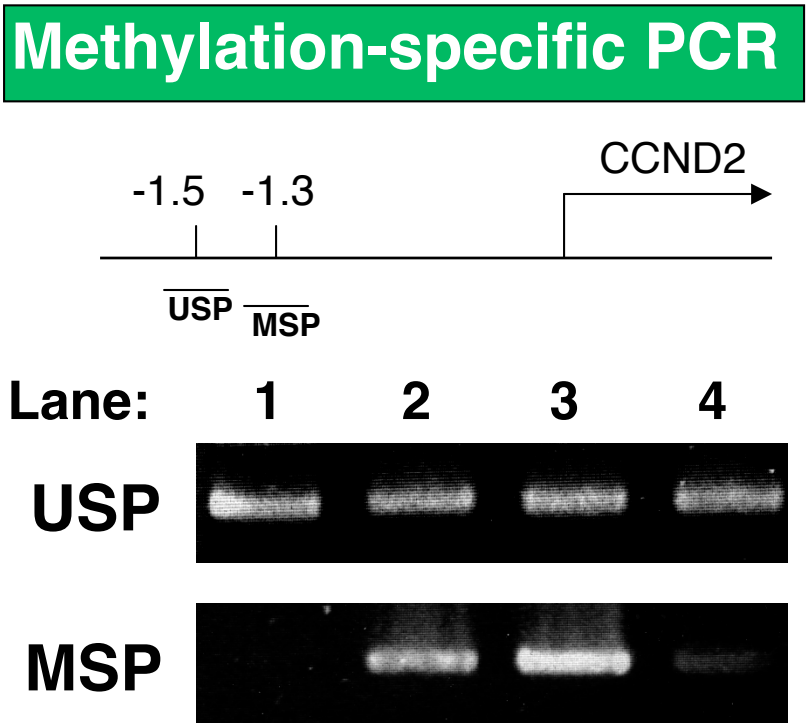

Figure 4. EZH2 knockdown leads to loss of DNA methylation within the CCND2 upstream region. The map depicts the CCND2 upstream region, which contains CpG islands in the -1.3 to -1.5 kb region known to be methylated in breast cancer cells. Methylation-specific PCR was used to detect CpG DNA methylation in genomic DNA isolated from SKBR3 cells. "USP" indicates a pair of PCR primers designed to detect unmethylated CpG DNA in the -1.5 kb region and "MSP" indicates a second pair of PCR primers to detect methylated CpG DNA in the -1.3 kb region. Accumulation of PCR products is shown from reactions using the USP and MSP primers, as indicated, and the following templates: untreated CCND2 upstream fragment (lane 1), CCND2 upstream fragment pre-methylated by SssI methylase (lane 2), genomic DNA from cells treated with control (non-target) RNAi (lane 3), and genomic DNA from cells treated with EZH2 RNAi (lane 4). All templates were treated with bisulfite to convert unmethylated C residues to T residues prior to PCR. Comparison of MSP signals in lanes 3 and 4 indicates that EZH2 is required for CpG DNA methylation in the CCND2 upstream region.

Figure 5

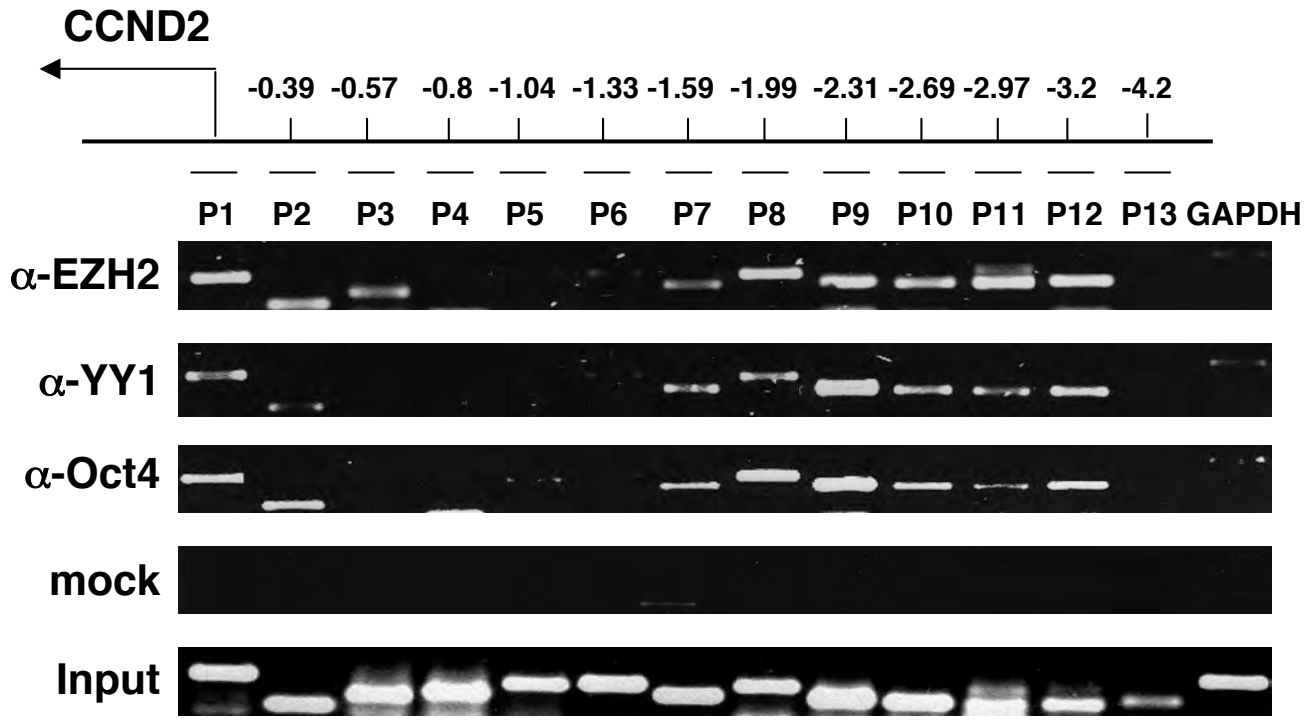

Figure 5. Mapping of EZH2, YY1, and OCT4 chromatin association within the CCND2 upstream region. Chromatin IPs were performed on SKBR3 cells, with successive primer pairs used to map indicated regulatory proteins within the 4 kb region upstream of the CCND2 start site (arrow). Top three panels show association of EZH2, YY1 and OCT4, as indicated. The fourth panel shows a mock IP negative control and the fifth panel shows PCR products amplified from input genomic DNA. GAPDH is a negative control gene which shows little or no association with these regulatory factors.

Figure 6

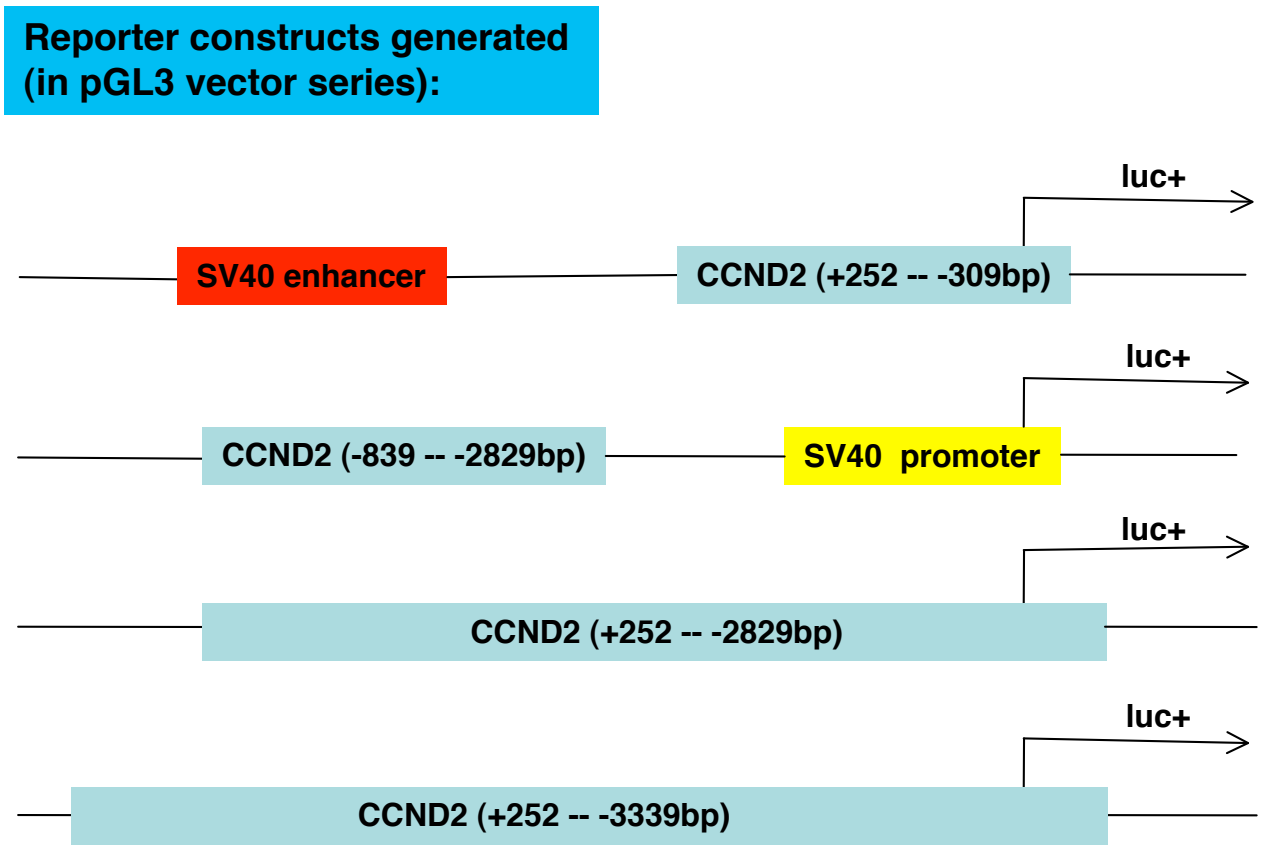

Figure 6. Reporter constructs to test for EZH2 silencing in breast cancer cells. All constructs contain regulatory DNA fused to the coding region of the firefly luciferase gene (luc+). The bottom two constructs contain intact segments of CCND2 upstream DNA that span the major regions of EZH2 association. The top two constructs are composites containing either the CCND2 promoter region or an isolated CCND2 upstream fragment in combination with the SV40 enhancer or promoter.